JOURNAL OF BACTERIOLOGY, Dec. 1972, p. 1118-1126

Vol. 112, No. 3
Printed in U.S.A.

Biosynthetic Pathway of Thiamine Pyrophosphate: a Special Reference to the Thiamine Monophosphate-Requiring Mutant and the Thiamine Pyrophosphate-Requiring Mutant of *Escherichia coli*

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Received for publication 31 August 1972

Two types of mutants of *Escherichia coli* were isolated, one of which (mutant 70-23-107) responded to thiamine pyrophosphate, and the other (mutant 70-23-102) to thiamine monophosphate and thiamine pyrophosphate. They were produced by further mutation of a thiamine auxotroph of *E. coli* 70-23 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The parent organism required thiamine because phosphohydroxymethylpyrimidine kinase activity was lacking in this organism, and hydroxymethylpyrimidine pyrophosphate was not permeable through the cell membrane of *E. coli*. Thiamine, thiamine monophosphate, and thiamine pyrophosphate were all equally active for the parent, whereas mutants 70-23-102 and 70-23-107 lost their ability to grow on thiamine. Both mutants differed only in the growth response to thiamine monophosphate: the former could grow on thiamine monophosphate, whereas the latter could not. Experimental results with the newly isolated mutants indicate that in *E. coli* the free form of thiamine is not involved in de novo synthesis of thiamine pyrophosphate, but thiamine monophosphate, an exclusive product formed by the reaction between hydroxymethylpyrimidine pyrophosphate and hydroxyethylthiazole monophosphate, is directly phosphorylated to form thiamine pyrophosphate. Exogenous thiamine, on the other hand, is converted to thiamine pyrophosphate via the intermediate formation of thiamine monophosphate.

It has been thought that in *Escherichia coli* thiamine pyrophosphate (TPP) is synthesized from thiamine monophosphate (TMP) by a pathway similar to that proposed for bakers' yeast (9, 15); yeast synthesizes TPP from TMP via the intermediate formation of the free form of thiamine (3). However, successful isolation of two kinds of *E. coli* mutants, which were unable to grow on thiamine but did grow on TMP and TPP, brought us to a number of questions on the proposed mechanism on TPP synthesis. Our previous results obtained with these mutant strains have indicated that TPP is synthesized from TMP and that free thiamine is not involved as an intermediate in de novo synthesis of TPP. Exogenous thiamine, on the other hand, is utilized by the cells and is converted to TPP via the intermediate formation of TMP (13). This paper adds further evidence that supports the previous conclusions.

MATERIALS AND METHODS

Organisms. *E. coli* W (referred to as original strain) and its derivatives were used. Mutant 70-23 (parental strain) was obtained from B. D. Davis of Harvard Medical School in 1955 and has been maintained in our laboratory. The biochemical lesion of this organism was thought to be at the stage in the phosphorylating step of 2-methyl-4-amino-5-hydroxymethylpyrimidine monophosphate (hydroxymethylpyrimidine-P); hydroxymethylpyrimidine pyrophosphate (hydroxymethylpyrimidine-PP) is not formed from the monophosphate. Thiamine is required by this organism as the intact molecule because hydroxymethylpyrimidine-PP is not permeable through the cell membrane (10, 12). Mutants reported here were isolated by treatment of mutant 70-23 with

N-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method reported by Adelberg, Mandel, and Chen (1). The treated cells were cultured at 37 C for 24 hr in a minimal medium supplemented with 0.1 μM TPP. Prototrophic and parental types of cells were eliminated in a minimal medium containing 0.01 μM thiamine and 200 IU of penicillin per ml. Mutants that could grow on TPP, but not on thiamine, were selected by the replica plating method of Lederberg and Lederberg (7). Two mutant strains showing such growth responses were isolated, but they showed different growth response to TMP: one (mutant 70-23-102) responded to both TMP and TPP, whereas the other (mutant 70-23-107) responded to TPP but not to TMP. Mutants 102-R1, 102-R2, and 102-R3 were revertants obtained from mutant 70-23-102. These revertants were obtained as described in Results.

Media and growth conditions. For growth experiments, the minimal medium of Davis and Mingioli (4) was used as basal medium. Solid medium contained 1.5% washed agar. For batch culture, a 1-liter culture that had been aerated by shaking was used as an inoculum for 30 liters of the same medium aerated by air aspiration. The medium was supplemented with 0.01 μM TPP. After 20 hr, the cells were harvested and washed twice with 0.02 M cold potassium phosphate buffer (*p*H 7.2) containing 1 mM $MgCl_2$. The pellet was used as a source of cell suspensions and crude extracts.

Preparation of crude extracts. A 5-g amount of the cell paste was suspended in 15 ml of 0.02 M potassium phosphate buffer (*p*H 7.0) that contained 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol. In some experiments, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, was used instead of the phosphate buffer. The cells in the suspension were disrupted by sonic oscillation (20 kc) for 3 min, and the resulting suspension was centrifuged at 10,000 $\times$ *g* for 10 min. The supernatant fraction was dialyzed against 2 liters of the same buffer containing 1 mM $MgCl_2$ and 0.1 mM β-mercaptoethanol with two changes. The dialyzed fluid, referred to as a crude extract, containing approximately 40 mg of protein per ml, was used to measure the phosphorylating activities of thiamine.

Paper chromatographic and bioautographic methods. Paper chromatograms were developed by the ascending technique. The solvents used were (A) isopropyl alcohol-0.3 M (*p*H 7.0) potassium phosphate buffer (2:1, v/v), and (B) isopropyl alcohol-1 M (*p*H 5.0) acetate buffer-water (7:1:2, v/v/v). The R_F values of thiamine, TMP, and TPP were 0.60, 0.24, 0.08, respectively, by solvent A, and 0.55, 0.30, 0.18, respectively, by solvent B. Bioautography was used to estimate the extent and the rate of formation of TMP from thiamine and that of TPP from TMP. For this purpose, a 300-ml portion of the minimal medium containing 1.5% agar was melted and cooled in a water bath to 50 C. A 3.0-ml amount of bacterial suspension (ca. 10^9 cells per ml) was added into the agar, which was then poured into a sterile, glass dish (300 by 200 by 5 mm). The developed chromatogram was air-dried and placed in contact with the surface of the solid medium. The chromatogram was removed after 10 min, and the plate was covered and incubated at 37 C for 20 hr. The inoculum used to seed the plates in these experiments consisted of the suspension of washed cells taken from a 24-hr culture of the test organism grown on the minimal agar slant containing 0.05 μM TPP. Mutant 70-23 was used as a test organism to detect thiamine, TMP, and TPP. In some experiments, mutant 70-23-107 was used to confirm TPP present in the chromatogram.

Chemicals. Thiamine, TMP, and TPP were purchased from Sigma Chemical Co. Bioautographic examination revealed that the preparation of TMP contained a small amount of free thiamine, and that TPP also contained free thiamine and TMP, each in a small amount. Therefore, these preparations were purified by column chromatography by using an anion exchange resin (Dowex 50W) before use.

RESULTS

Growth responses of newly isolated mutants. On solid media containing either thiamine, TMP, or TPP, parental strain 70-23 gave colonies of the same size after 24 hr of incubation. Mutant 70-23-102 was able to grow on either TMP or TPP, whereas mutant 70-23-107 grew on TPP but not on TMP. Both mutants gave no visible growth on thiamine even after 3 days of incubation. To permit quantitative comparison of the strains, their growth responses in liquid media were also tested. The growth of mutant 70-23-102 was proportional to the amount of TPP supplemented into the medium at the concentration ranging from 1 nM to 100 nM (Fig. 1). The TPP requirement for this organism was replaceable by the same amount of TMP, whereas thiamine had no effect even in the presence of higher concentration or after prolonged incubation. In the case of mutant 70-23-107, growth was permitted only by TPP, although the amount of TPP required for a half-maximal growth was somewhat more than that required by mutant 70-23-102. The different growth responses of the parent and mutant organisms with thiamine and its phosphate esters appear to be due to the second mutation which affects the phosphorylating steps of thiamine.

TPP synthesis by cell suspensions. Freshly prepared cell suspension of each organism was incubated with free thiamine or TMP in the presence of glucose as energy source, which is thought to be necessary in both uptake and phosphorylating processes of thiamine by intact cells. The complete reaction system contained: cell suspension (10 mg [dry weight]/ml), 0.3 ml; 1 mM thiamine or TMP, 0.1 ml; 4% glucose, 0.1 ml; 1 M potassium phosphate buffer (*p*H 7.0), 0.2 ml; 0.1 M $MgCl_2$, 0.1 ml;

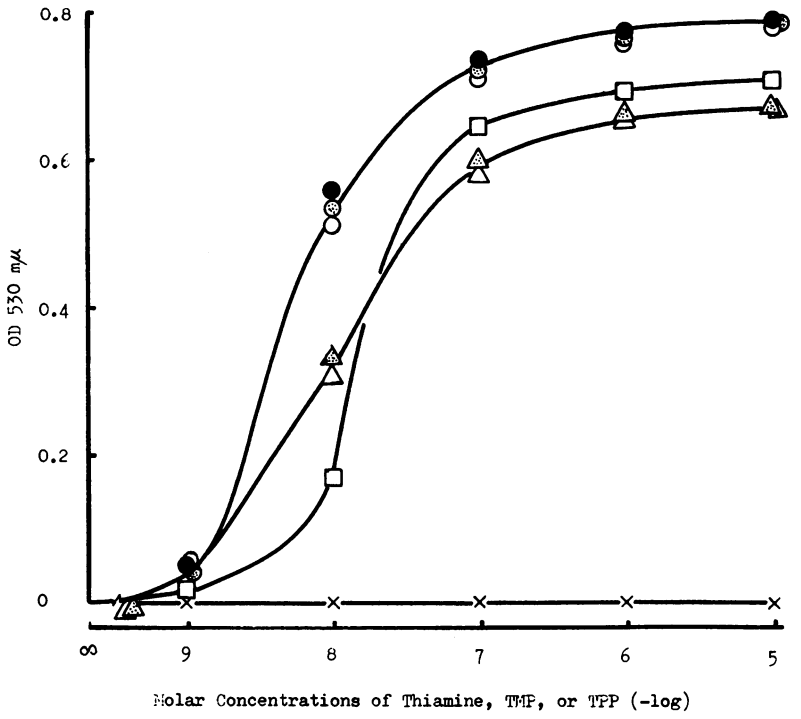


FIG. 1. *Growth responses of parental and mutant organisms to thiamine or its phosphate esters under aerobic conditions. The cells, harvested from a culture growing aerobically on a limiting amount of TPP, were washed and then suspended in the growth medium of Davis and Mingioli. Growth responses of mutant 70-23 (parental strain) to thiamine (●), TMP (◑), or TPP (○); growth responses of mutant 70-23-102 to TMP (▲) or TPP (△); growth response of mutant 70-23-107 to TPP (□); growth responses of mutant 70-23-102 to thiamine and mutant 70-23-107 to thiamine or TMP (×). Optical density was measured at 530 nm after 36 hr of incubation at 37 C, with shaking.*

and water, 0.2 ml. After the reaction was carried out at 37 C for 1 hr in a shaking water bath, each tube was adjusted to *p*H 4.5 by N HCl and heated at 80 C for 20 min. The tubes were centrifuged, and 10 μliters of this clear supernatant fraction was spotted on paper (Tōyo filter paper no. 50) and developed with solvent A. Thiamine, TMP, and TPP were located by bioautography with mutant 70-23 as a test organism. Sizes (average diameter) of the growth zones of this organism given by each thiamine compound is proportional to a logarithm of the amount of these compounds spotted on the paper. Therefore, it is possible to estimate the amount of thiamine, TMP, or TPP by comparison with the size of the growth zones derived from standard amount of corresponding thiamine compound spotted on the chromatograms. The results of the formation of TPP from TMP and from thiamine are presented in Table 1. In reaction mixtures containing suspensions of either strain W or mutant 70-23, TMP and TPP were formed from thiamine, and TPP from TMP. A far greater amount of TPP than of TMP was formed from thiamine as a substrate. This indicates that the enzymatic activity responsible for the conversion from thiamine to TPP was highly active in intact cells and that TMP arose intermediately only as a transitory existence. As for the cells of mutant 70-23-102, neither TMP nor TPP was formed from free thiamine, whereas TPP was formed from TMP, though in a small amount. The uptake ability of these mutant cells for either thiamine or TMP, as compared with the parental cells, apparently decreased (data not shown). This would explain why the mutant cells showed low activity of the conversion from TMP to TPP. These results suggest that the organism is lacking in the enzymatic activity which catalyzes the conversion from thiamine to TMP. In addition, mutant 70-23-107 cells could catalyze the reaction from free thiamine to TMP, whereas TPP was not formed from either thiamine or TMP; the enzymatic step catalyzing from TMP to TPP is blocked in this

TABLE 1. *Phosphorylation of thiamine or thiamine monophosphate (TMP) by cell suspensions prepared from original, parental, and mutant organisms*

Bacterial strains	Substrates	Thiamine phosphates formed[a]	
		TMP	TPP
W	Thiamine	0.14[b]	2.18
W	TMP		2.64
70-23	Thiamine	0.10	2.58
70-23	TMP		5.30
70-23-102	Thiamine	0	0
70-23-102	TMP		0.82
70-23-107	Thiamine	1.66	0
70-23-107	TMP		0

[a] Cell suspensions prepared from each organism grown on a limiting amount of TPP were incubated with thiamine or TMP under the presence of glucose at 37 C for 1 hr. The amounts of TMP and TPP formed were estimated by microbiological assay with mutant 70-23 as described in Results.

[b] Nanomoles per milligram (dry weight) of cells per hour.

organism. These results support the assumption that TPP can be formed stepwise from free thiamine via the intermediate production of TMP in *E. coli* cells.

Formation of TMP from thiamine by cell extracts. Crude extract prepared from parental and mutant organisms was used as an enzyme preparation. Sonic oscillation and dialysis were carried out in the potassium phosphate buffer (see Materials and Methods). The complete system contained: crude extracts, 0.25 ml (10 mg of protein); 0.1 mM thiamine, 0.1 ml; 0.1 M adenosine triphosphate (ATP), 0.1 ml; 1 M potassium phosphate buffer (*p*H 7.0), 0.1 ml; 0.1 M $MgCl_2$, 0.1 ml; and water, 0.35 ml. The reaction was carried out at 37 C for 30 min. After the reaction was over, the reaction mixture was adjusted to *p*H 4.5 by 0.2 N HCl, heated at 90 C for 5 min, and centrifuged. A 10-μliter amount of the resulting supernatant fraction was spotted on the paper and developed by solvent B. Thiamine, TMP, and TPP were located by bioautography with mutant 70-23. Crude extracts prepared from original, parental, and mutant 70-23-107 cells could catalyze the reaction from thiamine to TMP, whereas extract from mutant 70-23-102 could not (Fig. 2), i.e., mutant 70-23-102 is lacking in the enzymatic activity of thiamine monophosphokinase which catalyzes the conversion from thiamine to TMP. These results indicate that the conversion of thiamine to TMP is readily catalyzed by the crude extracts, but the conversion of TMP to TPP is not likely to occur under the experimental conditions, and that the overall reaction from thiamine to TPP stops at the stage up the formation of TMP. This is in contrast to the observation that the overall reaction proceeds rapidly by intact cells, and only a small amount of TMP appears as an intermediary product.

Formation of TPP by cell extracts. A small amount of TPP was contained in the preparation of crude extracts obtained from the cells grown with a limiting amount of TPP. Preliminary experiments revealed that such TPP might be enzyme bound and was difficult to remove by dialysis even after ammonium sulfate fractionation under alkaline conditions. Moreover, when reaction mixtures containing crude extracts, but not substrate, were incubated at 37 C, the TPP content gradually decreased, with the concomitant formation of

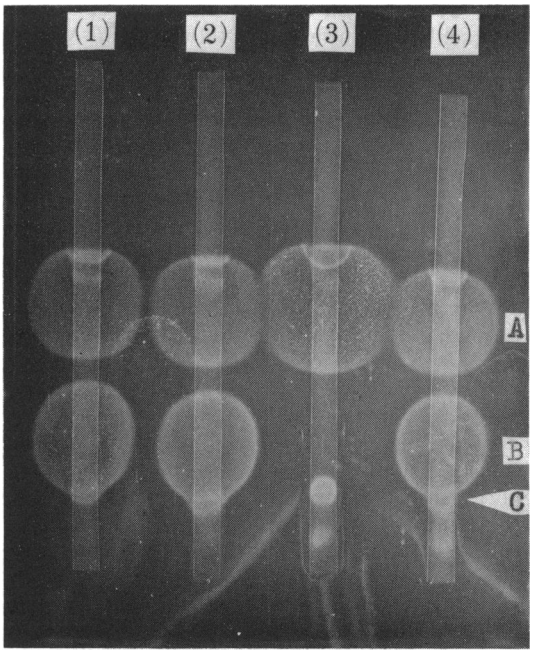


FIG. 2. *Separation of the enzymatically synthesized TMP from thiamine by paper bioautography with mutant 70-23 as a test organism. Cell extracts prepared from (1) strain W, (2) mutant 70-23, (3) mutant 70-23-102, and (4) mutant 70-23-107 were incubated at 37 C for 30 min with thiamine and ATP under the conditions described in Results. Solvent used was isopropyl alcohol-1 M (pH 5.0) acetate buffer-water (7:1:2, v/v/v). Direction of migration of thiamine compounds was from bottom to top. A, Thiamine; B, TMP; and C, TPP.*

TMP. When a prolonged incubation was performed, all of the TPP was converted to thiamine. This apparent phosphatase activity proceeded much more rapidly under acidic conditions than under alkaline conditions. Such an activity might obscure TPP formation in an in vitro system.

It has been reported that the requirement for thiamine in the parent strain could be replaced by succinate under anaerobic conditions (11, 14). Although the reason for this response had not yet been explained entirely, only a one-tenth amount of TPP was formed in the parental cells grown on succinate compared with the cells grown under the presence of a limiting amount of thiamine. Therefore, we thought that such TPP-deficient cells might be available as a source of crude extracts for examination of the formation of TPP formed in minute amounts. For this purpose, mutant 70-23 was grown anaerobically in the medium which contained 0.2 mM sodium succinate and without thiamine, and was harvested by centrifugation. The cells were washed and disrupted in the Tris-hydrochloride buffer (see Materials and Methods). The disrupted suspension was centrifuged, and the clear supernatant fraction, without dialysis, was used as crude extract. The complete system contained: crude extract, 0.4 ml (10 mg of protein); 1 mM thiamine or 1 mM TMP, 0.1 ml; 1 M Tris-hydrochloride buffer (*p*H 7.5), 0.1 ml; 0.1 M ATP, 0.1 ml; 0.1 M $MgCl_2$, 0.1 ml; 2 mM 1-cysteine, 0.1 ml; and water, 0.1 ml. After the reaction had proceeded at 37 C for 2.5 hr, 10 μliters of reaction mixtures was immediately placed on the chromatographic paper and was developed by solvent A. Thiamine, TMP, and TPP were located by bioautography with mutant 70-23.

That thiamine phosphates were formed with the enzyme preparation is shown by the tracing of the bioautograph in Fig. 3. Incubation of thiamine and ATP with the enzyme preparation resulted in the formation of both TMP and TPP. However, when TMP was used as a substrate together with ATP, TPP was formed in a larger amount than in the reaction mixture containing thiamine and ATP. When ATP was omitted, no appreciable TPP was produced, although the reaction from thiamine to TMP had occurred to some extent. Control experiments showed that there was no significant amount of TPP in the crude extract itself. These results further substantiate the idea that mutant 70-23 contains two different kinds of enzyme: one catalyzing the conversion from thiamine to TMP; and the other, the conversion

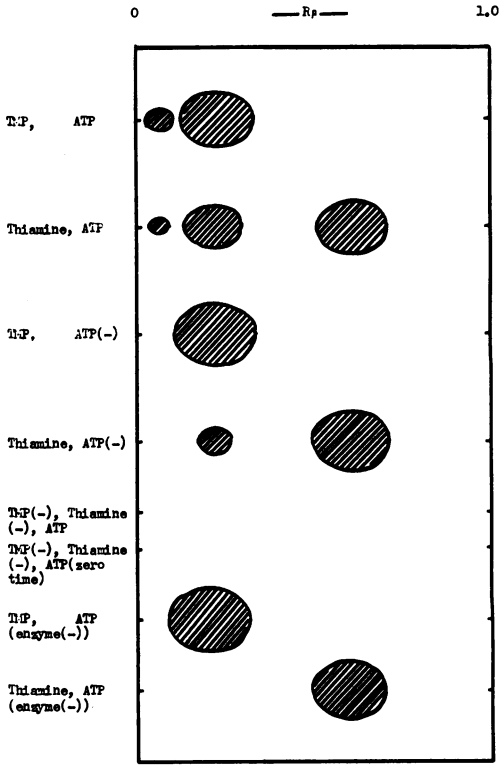


FIG. 3. *Separation of the enzymatically synthesized TPP from thiamine or TMP by paper bioautography with mutant 70-23 as a test organism. Cell extracts, prepared from the cells of mutant 70-23 grown on succinate under anaerobic conditions, were incubated at 37 C for 2.5 hr with thiamine or TMP in the presence or absence of ATP. Solvent used was isopropyl alcohol-0.3 M (pH 7.0) potassium phosphate buffer (2:1, v/v). Detailed assay conditions were given in Results.*

from TMP to TPP. The amount of TMP formed from thiamine was, however, far greater than that of TPP formed from TMP or thiamine. Such was also the result in the previous experiments (13) in which crude extracts prepared from osmotically shocked cells of strain W and mutant 70-23 were used.

Properties of revertants obtained from mutant 70-23-102. An attempt has been made to select for spontaneous mutants reverted from mutants 70-23-102 and 70-23-107. Examination of revertants grown in a thiamine-enriched medium revealed that they always expressed as a phenotype similar to that of mutant 70-23. This observation indicated that double mutations had occurred in both mutants, one pertaining to the phosphorylation of hydroxymethylpyrimidine-P (original block) and the other

to the synthesis of TPP from thiamine. However, when minimal medium without thiamine was used for the selection, no spontaneous revertants appeared. These results led to the hypothesis that if the original block in mutant 70-23-102 were removed by a reverse mutation the strain should be a thiamine prototroph although it would still lack thiamine monophosphokinase. Efforts were made, therefore, to isolate such a partial revertant by ultraviolet light mutagenesis.

For ultraviolet irradiation, a 20-w Toshiba Germicidal Lamp was used. Mutant 70-23-102 cells grown in the minimal medium supplemented with 0.02 μM TPP were diluted 100 times in sterile water. A 10-ml amount of the bacterial suspension was exposed in an open petri dish about 40 cm from the lamp for 30 sec in a dark room. A 1.0-ml amount of the irradiated suspension was transferred to the minimal medium containing TMP and incubated at 37 C for 16 hr. A portion of this culture was streaked on a minimal agar plate, and colonies that grew rapidly on the plate were isolated. Pure cultures were obtained by reisolating the colonies on the plate which contained TMP. Three strains of the revertants (102-R1, 102-R2, and 102-R3), each derived from individual irradiation experiments, were thus produced. The fact that the growth on the minimal medium of these revertants was comparable to that of W strain afforded indirect evidence that the original block had been removed by a back-mutation. However, more direct evidence was required to ascertain whether these revertants had lost the original block but still lacked thiamine monophosphokinase activity.

The latter activity was assayed by the method essentially like that employed for TPP synthesis by cell suspensions as described above. Since the uptake of thiamine into the cells was of primary importance in the phosphorylation of thiamine by intact cells, the following modifications were made. After the reaction was over, each tube was centrifuged and the cells were washed once by 5.0 ml of cold 0.2 M potassium phosphate buffer, *p*H 7.0, by centrifugation. Thiamine and its phosphate esters were then extracted from the cells by heating at 80 C for 20 min in 1.0 ml of 0.2 M acetate buffer, *p*H 4.5.

To examine the activity of phosphohydroxymethylpyrimidine kinase, intact cells of original, parental, and revertant strains were compared for their abilities to catalyze TPP-forming reactions from the pyrimidine and thiazole moieties of thiamine. Our experience has indicated that in *E. coli* both hydroxymethylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole), supplied from outside, are taken up into the cells and converted to TPP through the de novo synthetic pathway. The phosphorylation of hydroxymethylpyrimidine-P is involved as an essential step in a series of the reaction. The complete system contained: cell suspension (10 mg [dry weight]/ml), 0.3 ml; 1 mM hydroxymethylpyrimidine, 0.1 ml; 1 mM hydroxyethylthiazole, 0.1 ml; 10% glucose, 0.1 ml; 1 M potassium phosphate buffer (*p*H 7.0), 0.2 ml; 0.1 M $MgCl_2$, 0.1 ml; and water, 0.1 ml. After the reaction had proceeded at 37 C for 1 hr in a shaking water bath, each tube was adjusted to *p*H 4.5 with N HCl and heated at 80 C for 20 min. Thiamine and its phosphate esters were assayed by paper bioautography with solvent B.

The results (Table 2) showed that both the original and parental organisms were able to take thiamine into the cells and convert it into TPP, whereas free thiamine was accumulated in the revertant cells without conversion to the phosphate esters. In the reaction mixture containing the pyrimidine and thiazole moieties, neither TMP nor TPP was formed by mutant 70-23 cells, whereas TPP was synthesized in a significant amount by the cells of strain W and the revertants. These results showed clearly that the restoration from the de novo block and the ability to utilize exogenous thiamine by the revertants were not related phenomena.

Growth inhibition of mutant 70-23-102 by thiamine and of mutant 70-23-107 by either thiamine or TMP. Growth inhibition of mutant 70-23-107 by TMP was first observed in solid medium. When a developed, paper chromatogram containing both TMP and TPP was placed on the surface of the bioautographic plate with mutant 70-23-107 as a test organism, the growth zone corresponding to the R_F value of TPP always showed a crescent shape, which indicated inhibition by a component migrating farther than TPP. Experiments in which thiamine, TMP, and TPP were supplied by placing three small crystals on the same bioautographic plate each, in order to form the vertex of a triangle, revealed that the TPP-dependent growth of mutant 70-23-107 was inhibited to the same extent by both thiamine and TMP. The growth inhibition of mutant 70-23-102 by thiamine was also demonstrated by the same type of experiment. To measure a quantitative growth inhibition of both mutants, a liquid medium was used in the subsequent experi-

TABLE 2. *Thiamine phosphates formation[a] from thiamine or its pyrimidine and thiazole moieties by cell suspensions prepared from revertants of mutant 70-23-102*

Bacterial strains	Thiamine accumulated[b] with thiamine substrate	Thiamine and thiamine phosphates formed[b]				
		Thiamine substrate		Pyrimidine[c] + thiazole[d] substrate		
		TMP	TPP	Thiamine	TMP	TPP
70-23	2.52	0.98	3.13	0	0	0
W	1.05	0.34	2.67	0	0.01	0.20
102-R1	5.80	0	0	0	0.01	0.21
102-R2	5.37	0	0	0	Trace[e]	0.16
102-R3	6.42	0	0	0	Trace	0.16

[a] Cell suspensions prepared from each organism were incubated with thiamine or its moieties under the presence of glucose at 37 C for 1 hr. The amounts of thiamine accumulated in the cells, and thiamine phosphates formed were estimated by microbiological assay with mutant 70-23 as described in Results.

[b] Nanomoles per milligram (dry weight).

[c] 2-Methyl-4-amino-5-hydroxymethylpyrimidine.

[d] 4-Methyl-5-β-hydroxyethylthiazole.

[e] The amount of TMP formed was less than 0.01 nmoles per mg (dry weight) per hour.

ments. For this purpose, in the case of mutant 70-23-107, for example, three series of the minimal medium were supplemented with 1 μM, 0.1 μM, and 0.01 μM TPP, respectively. Thiamine or TMP, at a final concentration ranging from 0.1 mM to 0.01 μM, was added to a series of tubes which contained the same amount of TPP. Cells of mutant 70-23-107 from a 24-hr culture in medium containing 0.01 μM TPP were washed and inoculated into the medium to yield the final concentration of 10^5 cells/ml. After incubation for 36 hr at 37 C with shaking, growth was measured by optical density at 530 nm. TPP-dependent growth of mutant 70-23-107 was inhibited by either thiamine or TMP to the same extent; the amount of thiamine or TMP required for complete inhibition was observed to be approximately 10 times greater than that of TPP (Fig. 4).

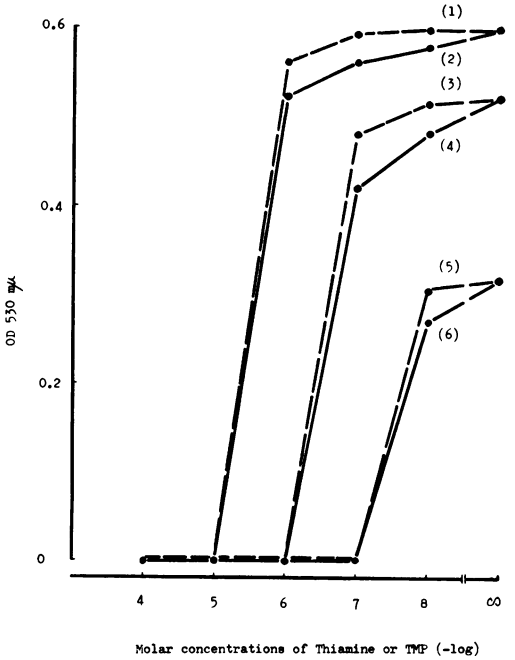
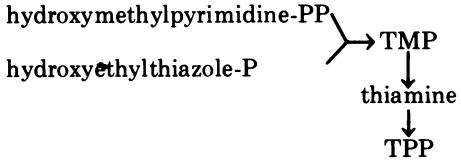


FIG. 4. *Growth inhibition of mutant 70-23-107 by either thiamine or TMP. The cells, harvested from a culture growing aerobically on a limiting amount of TPP, were washed and then suspended in the growth medium which contained (1) 1 μM TPP plus varying amounts of TMP, (2) 1 μM TPP plus varying amounts of thiamine, (3) 0.1 μM TPP plus varying amounts of TMP, (4) 0.1 μM TPP plus varying amounts of thiamine, (5) 0.01 μM TPP plus varying amounts of TMP, and (6) 0.01 μM TPP plus varying amounts of thiamine. Optical density was measured at 530 nm after 36 hr of incubation at 37 C with shaking.*

DISCUSSION

It has generally been assumed that thiamine is a precursor of TPP in certain species of microorganisms including yeast and *E. coli*, because in these organisms intracellular accumulation of TPP is significantly increased when thiamine is added to the medium. An enzyme that catalyzes the formation of TPP from thiamine by a direct pyrophosphoryltransfer from ATP has been purified from yeast (5, 16) and named thiamine pyrophosphokinase (ATP: thiamine pyrophosphotransferase, EC 2.7.6.2). This enzyme has been assumed to be involved in the de novo synthesis of TPP. The reaction sequences implicated in the biosynthesis of TPP has been shown by Camiener and Brown

(2, 3, 8), who used bakers' yeast as an experimental material. The proposed pathway is:



However, our experimental results obtained with newly isolated mutants of *E. coli* that require TMP or TPP cannot be explained by this pathway.

It has been reported that in mutant 70-23 cells a minute amount of TPP was synthesized under the conditions in which excess succinate was present intracellularly (11). Succinate is active for mutant 70-23-102 in the absence of TMP but not for mutant 70-23-107 (data not shown). This suggests that TMP and, hence, TPP are possibly synthesized de novo in the cells of mutant 70-23-102 under the conditions where succinate is available but thiamine cannot proceed to TMP. Furthermore, mutant 70-23-102 was irradiated with ultraviolet light to increase the rate of reversion back to the prototrophic state, and three strains of such revertants could be isolated. Each one proved to have lost the de novo block (original block), but they were still lacking in thiamine monophosphokinase activity. These results are entirely consistent with our conclusion: free thiamine is not involved in the de novo synthesis of TPP. Experimental results obtained with cell suspensions and cell extracts of parental and mutant organisms lead to the conclusion that TPP is synthesized from thiamine via the intermediate formation of TMP, i.e., TMP occupies a position where exogenous thiamine joins with the de novo synthetic pathway for TPP. The metabolic steps which are blocked genetically in the mutant strains can be summarized as in Fig. 5.

Recently, the thiamine uptake system in *E. coli* has been examined, and it was reported that thiamine is transported into the cell by an energy-dependent process in which thiamine pyrophosphokinase in the cell membrane participates in the accumulation and concomitant formation of TPP (6). The thiamine pyrophosphokinase of *E. coli* should be separated into two different enzymes, thiamine monophosphokinase (or thiamine kinase) and thiamine monophosphate kinase. Therefore, the question arises as to whether thiamine monophosphokinase is involved in the transport system because exogenous thiamine is first converted into TMP by this enzyme. Such possibility, however, has been ruled out by the experiments in which ^{14}C-thiamine was incubated with the intact cells of mutant 70-23-102 (13). Therefore, it would be reasonable to assume that thiamine monophosphokinase is not involved as an essential component in the thiamine transport system. There remains, however, the possibility that the monophosphokinase facilitates the process of accumulation in the cells.

An interesting problem has been presented by the findings that the TMP-dependent growth of mutant 70-23-102 was competitively inhibited by thiamine and that TPP-dependent growth of mutant 70-23-107 was inhibited well by either TMP or thiamine. The inhibition occurred by the normal, structurally related metabolites arising at the sequence prior to the blocked reactions. One of the most possible explanations of this phenomenon is that thiamine and TMP can combine to the TPP-binding site of the apoenzyme(s) for which TPP can

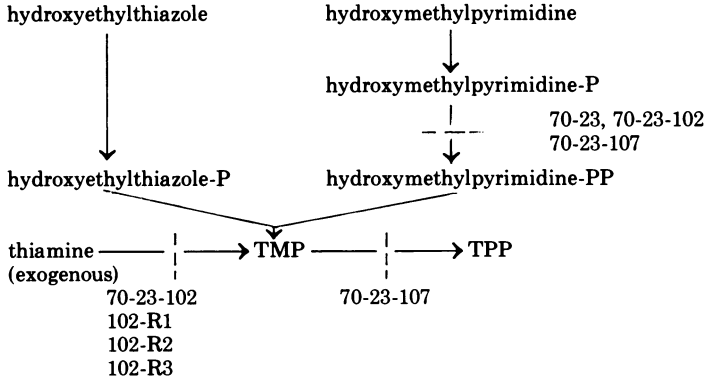


FIG. 5. *Synthesis of TPP from thiamine via the intermediate formation of TMP, in mutant strains of E. coli.*

serve as a coenzyme and that competition has occurred at the binding site. However, the TPP-dependent growth of mutant 70-23 was not inhibited by TMP or thiamine, and TMP-dependent growth was not inhibited by thiamine. This indicates that, in the parental cells, thiamine and TMP supplied from outside are phosphorylated to TPP and then associated onto the apoenzymes. The possibility, however, still exists that thiamine or TMP might be phosphorylated even after their association onto the apoenzymes. Further experiments are required to ascertain this hypothesis.

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